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Campylobacter jejuni Induces Secretion of Proinflammatory Chemokines from Human Intestinal Epithelial Cells

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Campylobacter jejuni **is a common cause of diarrhea in humans. While the pathogenic mechanisms of *C. jejuni* are not completely understood, host inflammatory responses are thought to be contributing factors. In this report, *C. jejuni* 81-176 is shown to up-regulate chemokines essential to inflammatory responses. Growth-related oncogene α (GROα), GROγ, macrophage inflammatory protein 1, monocyte chemoattractant protein 1 (MCP-1), and gamma interferon-inducible protein 10 (γIP-10) mRNA transcription in INT-407 cells was enhanced within 4 h of bacterial exposure. Infection with viable campylobacters was necessary for sustained chemokine transcription and was NF-κB dependent. GROα, γIP-10, and MCP-1 chemokine secretions were confirmed by immunological assays.**

Campylobacters are a leading cause of diarrheal disease worldwide (2), yet very little is known about bacterial pathogenesis or bacterium-host interactions. Inflammation of the colon is a hallmark of campylobacter infections, and leukocytes and erythrocytes are almost always found in stool during active illness (13). Inflammation is thought to mediate, at least in part, host injury (1).

Intestinal epithelial cells constitute one of the first physical barriers to enteric pathogens and likely initiate the host response. In response to injury, epithelial cells secrete cellular factors that are capable of recruiting macrophages and other cellular components of the immune and inflammatory responses (6). During campylobacter infections, mononuclear phagocytes infiltrate the submucosal lining as a consequence of tissue injury (12). Additionally, human epithelial and monocytic cell lines liberate potent proinflammatory cytokines (interleukin-6 and interleukin-8) in response to *Campylobacter jejuni* exposure in vitro (4, 5). We demonstrate here that epithelial cells transcribe and secrete other key chemokines essential to the activation of the host's inflammatory response when exposed to *C. jejuni* 81-176 (4).

GRO gene transcription. The growth-related oncogene α (GROα), GROβ, and GROγ chemokines are potent neutrophil chemoattractants produced by epithelial cells and a variety of other cell types (9, 10, 14). Expression of mRNA for these factors was assessed via reverse transcriptase (RT) PCR at 2, 4, and 24 h following infection of INT-407 cells with 81-176 (Fig. 1, lanes 2, 5, and 8). GROα message was slightly upregulated compared to uninfected controls at 2 and 4 h (Fig. 1, lanes 2 and 5). By 24 h, however, GROα mRNA transcription by cells cocultured with 81-176 was markedly enhanced compared to control cultures (lane 8). GROγ message was readily detectable in 81-176-inoculated culture wells at 2 and 4 h but was most prominent 24 h after infection (Fig. 1, lanes 2, 5, and 8). Epithelial cells cultured with tumor necrosis factor alpha (TNF-α) (20 ng/ml) served as positive controls for this assay and subsequent assays. GROβ message was not up-regulated by either 81-176 or TNF-α exposure but was detected in both uninoculated cultures and those cultured with campylobacters (Fig. 1, row 2).

Secretion of GROα by intestinal epithelial cells. The concentrations of GROα in supernatants of INT-407 cells were evaluated through enzyme-linked immunosorbent assay (ELISA) at 4 and 24 h after infection (Fig. 2A). Supernatants from 81-176-inoculated cultures demonstrated a slight increase in GROα levels (means ± standard deviations) compared to uninoculated culture wells as early as 4 h postinoculation (49 ± 76 pg/ml). However by the 24-hour time point, epithelial cells cocultured with 81-176 secreted 670 ± 81 pg/ml GROα ($P \leq 0.001$). TNF-supplemented cultures secreted 1,134 ± 163 pg/ml GROα at 4 h and 1,261 ± 284 at 24 h ($P < 0.001$). Chemokine levels detected in uninoculated controls were negligible at this time point (17 ± 30 pg/ml).

Transcription of MCP-1 and MIP-1α message. Monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1α (MIP-1α) are essential components of the immune response to enteric pathogens (3, 15). MCP-1 is a monocyte/basophil chemoattractant and activating factor produced by epithelial cells in response to physical assaults (7, 14). MIP-1α, a chemoattractant for B cells, eosinophils, and killer T cells, is produced by a variety of cell types (15). Within 2 hours of inoculation, MCP-1 message in tissue culture exposed to 81-176 was up-regulated. Substantial levels of MCP-1 message were detected in these cells by 24 h (Fig. 1, lanes 2, 5, and 8). MIP-1α message detected in 81-176-inoculated cultures remained at levels similar to those found in uninoculated control cultures, with only a slight up-regulation detected at 24 h (Fig. 1, lane 8). The TNF-α-treated cultures also demonstrated moderate induction of MIP-1α message compared to uninoculated cultures (Fig. 1, lane 9).

Secretion of MCP-1. The production of MCP-1 by epithelial cells was evaluated by ELISA at 4 and 24 h (Fig. 2B). INT-407 cells inoculated with 81-176 secreted moderate amounts of

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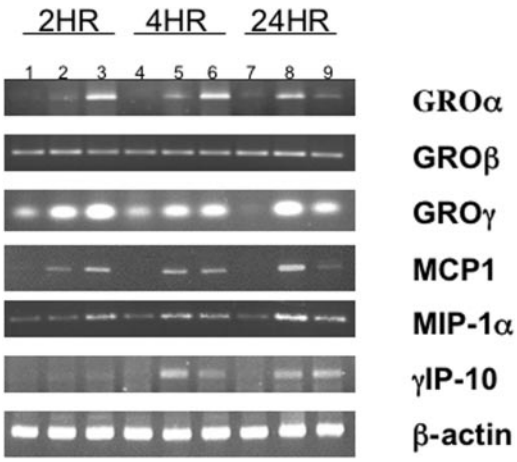

FIG. 1. Transcription of proinflammatory chemokine message by human intestinal epithelial cells to campylobacter exposure. INT-407 monolayers were inoculated with 81-176 at a 100:1 ratio of bacteria to epithelial cells. After 2, 4, and 24 h, total RNA was extracted by Trizol reagent (Invitrogen). Extracted RNA was transcribed into first-strand cDNA by using the ProSTAR first-strand RT-PCR kit (Stratagene). Negative controls were constructed by omitting RT or RNA (not shown). PCR primer pairs specific for human β-actin and chemokines were synthesized on an ABI 3400 DNA synthesizer as described previously (15). cDNA was amplified with *Taq* polymerase (Warrington, United Kingdom). β-Actin transcription is included as a housekeeping control in this experiment and subsequent experiments. Lanes 1, 4, and 7 show uninfected controls. Lanes 2, 5, and 8 show cells inoculated with 81-176. Lanes 3, 6, and 9 show the positive controls of the TNF-α assay.

MCP-1 at 4 h. Supernatants from cells cultured with 81-176 produced 128 ± 43 pg/ml MCP-1, compared to 6.2 ± 10 pg/ml detected in uninoculated cultures ($P \leq 0.01$). By 24 h, 793 ± 174 pg/ml MCP-1 was detected in 81-176-inoculated cultures, compared to 141 ± 68 pg/ml found in controls ($P \leq 0.003$).

Expression of interferon-inducible protein (γIP-10). Gamma interferon-inducible protein γIP-10 is a monocyte and T-lymphocyte chemoattractant and is frequently present in inflamed tissue (11, 15). γIP-10 message in INT-407 cells cocultured with 81-176 was up-regulated. Within 4 h of exposure to campylobacters, message was strongly up-regulated compared to that in uninoculated control wells, and levels of γIP-10 message remained elevated through 24 h (Fig. 1, lanes 5 and 8). INT-407 cultures supplemented with 20 ng/ml TNF-α up-regulated γIP-10 message within 4 h, and message remained elevated through 24 h, similar to results seen with 81-176-inoculated cultures (Fig. 1, lanes 6 and 9).

γIP-10 protein was detectable by ELISA in 24-hour cultures (Fig. 2C). Supernatants from 24-hour INT-407 cells cultured with 81-176 contained 1,356 ± 204 pg/ml γIP-10 ($P \leq 0.0004$). Normal or media control cultures produced negligible amounts of the cytokine (35 ± 61 pg/ml). TNF-α-supplemented cultures produced 1,862 ± 276 pg/ml of the chemokine by 24 h ($P \leq 0.0004$). At the earlier time point, there was no statistically significant difference in chemokine levels of 81-176 and TNF-α cultures compared to normal control cultures; *P* values were 0.98 and 0.493, respectively.

Viable campylobacters are required for sustained chemokine mRNA transcription. INT-407 monolayers were inoculated with 81-176, either viable or heat killed, at a multiplicity

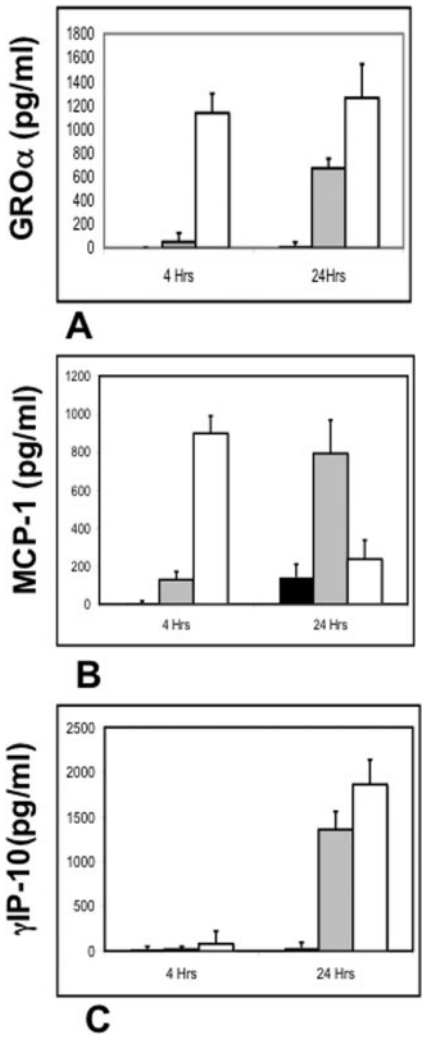

FIG. 2. Chemokine secretion by INT-407 cells following exposure to 81-176. INT-407 cells were cocultured with 81-176 at a 100:1 ratio of bacteria to epithelial cells as described previously (4). Cell culture supernatants were harvested at 4 and 24 h. Chemokine levels were assayed by ELISA per the manufacturer's instructions (R&D Systems). (A) Secretion of GROα. (B) Secretion of MCP-1. (C) γIP-10 secretion. Solid black bars, media control; solid grey bars, 81-176 coculture; white bars, TNF-α-supplemented cultures. Values (pg/ml) were calculated based upon standard curves generated with recombinant proteins. Cultures incubated with TNF-α served as a positive experimental control ($n = 3$). *P* values, in comparison to uninoculated control wells, are given in the text.

of infection of 100 bacteria to each epithelial cell. Heat-killed 81-176 was incubated at 70°C for 30 min and then plated to verify the absence of viability. To control for the effects of bacterial lipooligosaccharide (LOS)/lipopolysaccharide (LPS) on chemokine transcription, *Escherichia coli* LPS (Sigma Chemical Co., St. Louis, MO) was included in the assay at 50 μg/ml. Chemokine message was assayed at 4 and 24 h. Sustained GROγ, MCP-1, MIP-1α, and γIP-10 transcription required viable campylobacters or heat-sensitive bacterial products. Heat-killed 81-176 mediated a moderate and short-lived transcription of GROγ and MCP-1 genes that were detectable at 4 h but waned by the later time point compared to

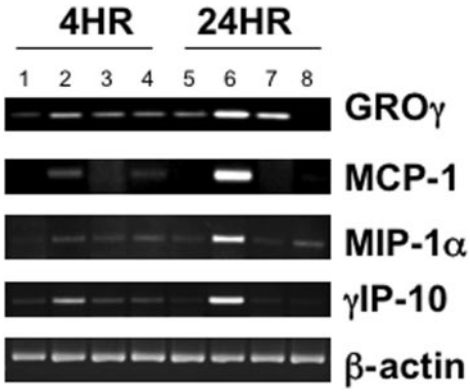

FIG. 3. Viable campylobacters are required for sustained chemokine mRNA transcription. INT-407 cells were cocultured with 81-176, either viable or heat killed, at a 100:1 bacteria-to-epithelial-cell ratio. To control for the possible effects of bacterial LOS/LPS on chemokine transcription, *E. coli* LPS was included in the assay at 50 μg/ml. RT-PCR was conducted as described in the legend for Fig. 1. Lanes 1 through 4, mRNA transcription at 4 h; lanes 5 through 8, chemokine RNA levels detected at 24 hours. β-Actin mRNA was assayed as a housekeeping control. Lanes 1 and 5, uninoculated controls; lanes 2 and 6, cultures incubated with viable 81-176; lanes 3 and 7, LPS control; lanes 4 and 8, cultures incubated with heat-killed 81-176. These data are representative of three independent experiments.

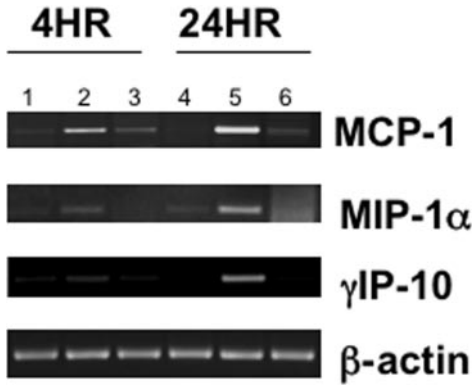

FIG. 4. Requirement of NF-κB activation for chemokine transcription. INT-407 monolayers were inoculated with viable 81-176 as described earlier. Transcription of chemokine genes was assayed as described earlier. Lanes 1 through 3, chemokine message detected at 4 h; lanes 4 through 6, message detected in 24-hour cultures. Lanes 1 and 4, uninoculated control cultures; lanes 2, 3, 5, and 6, mRNA transcription in cultures incubated with 81-176. Lanes 3 and 6, chemokine mRNA detected in cultures pretreated with the NF-κB inhibitor CAPE at 50 μM prior to inoculation with 81-176 (8). These data are representative of three independent experiments.

uninoculated control cultures (Fig. 3, lanes 1, 4, 5, and 8). A moderate MIP-1α response was also detected at 4 and 24 h (Fig. 3, lanes 4 and 8). With the exception of GROγ transcription, similar responses were observed in cultures incubated with *E. coli* LPS (Fig. 3, lanes 3 and 7). *E. coli* LPS induced GROγ transcription as late as 24 h, a response not detected when epithelial cells were inoculated with heat-killed campylobacters (Fig. 3, lane 7 and 8). Viable campylobacters induced marked transcription of each chemokine gene by 24 h (Fig. 3, lanes 2 and 6). These data indicate that viable 81-176 or possibly heat-sensitive or synthesized bacterial products other than LOS are required for inflammatory chemokine production by human epithelial cells.

Requirement of NF-κB activation for chemokine transcription. The requirement of transcription factor NF-κB activation for chemokine mRNA synthesis was evaluated through inhibition studies. Intestinal epithelial cells were preincubated with caffeic acid phenethyl ester (CAPE) (Sigma Chemical Co., St. Louis, Mo.) at 50 μM (8) for 1 h prior to inoculation with viable 81-176 at a multiplicity of infection of 100:1. CAPE is a potent and specific inhibitor of NF-κB activation and is known for its immunomodulatory and anti-inflammatory effects on a range of eukaryotic cell types (8). Preincubation of epithelial cells with CAPE prevented transcription of γIP-10 message at 4 and 24 h (Fig. 4, lanes 3 and 6). Inhibition of NF-κB activation also greatly reduced MCP-1 and MIP-1α transcription at both time points. These data suggest that transcription of the genes examined proceeds, at least in part, through the activation of transcription factor NF-κB. The incomplete inhibition of MCP-1 and MIP-1α might be due to the activity of compensatory transcription factors not affected by CAPE.

In summary, secretion of select chemokines by human intestinal epithelial cells exposed to campylobacters may provide the initial signals for acute inflammatory responses. We demonstrate that transcription of GROα, GROγ, MCP-1, MIP-1, and γIP-10 chemokine genes are up-regulated by human intestinal epithelial cells following exposure to viable 81-176 or bacterial products other than LOS. These transcriptional responses were apparently mediated by NF-κB activation. We also demonstrate the secretion of GROα, MCP-1, and γIP-10 proteins by ELISA. These chemokines are essential components of the inflammatory immune response to enteric pathogens, and additionally, these data provide insight into the mechanisms of tissue injury by campylobacters. These data further support the premise that inflammation plays a significant role in *C. jejuni* pathogenesis and that the intestinal epithelial tissue likely play significant roles in initiating the inflammatory response.

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